THE ROLE OF LIPOXYGENASES IN MEDIATING DEFENSE RESPONSE TO PERCEPTION OF HERBIVORE-INDUCED PLANT VOLATILES IN MAIZE

An Undergraduate Research Scholars Thesis

by

KATIE TOOMEY

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I, Katie Toomey, certify that all research compliance requirements related to this Undergraduate Research Scholars thesis have been addressed with my Research Faculty Advisor prior to the collection of any data used in this final thesis submission.

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ABSTRACT

The Role of Lipoxygenases in Mediating Defense Response to Perception of Herbivore-Induced Plant Volatiles in Maize

Katie Toomey
Department of Plant Pathology and Microbiology
Texas A&M University

Research Faculty Advisor: Dr. Mikhailo V. Kolomiets
Department of Plant Pathology and Microbiology
Texas A&M University

Volatile organic compounds (VOCs) are one means for inter- and intra-plant signal communication. One such group of volatiles produced in response to insect herbivory are herbivore-induced plant volatiles (HIPVs), which allow uninfested plants in close proximity to infested plants to pre-emptively raise their defenses to impending insect attack. Plant response to HIPVs involves the synthesis of various lipoxygenase (LOX)-derived oxylipins, including a well-known phytohormone that participates in insect defense, jasmonic acid (JA). Importantly, LOXs can collectively synthesize many diverse metabolites, however, whether any of these other oxylipins are involved in insect defense or HIPV response remain largely unknown. Green leaf volatiles (GLVs) are a major group of oxylipin HIPVs and are known to induce the expression of specific LOXs. Several maize knockout mutant lines disrupted in specific LOX genes were chosen because expression of those genes is GLV-inducible. Therefore, these mutants are hypothesized to lack appropriate defense metabolite responses to HIPVs. By selectively testing metabolite responses of *lox* mutants to HIPVs, we aim to identify LOX isoforms that are

involved in HIPV response and the specific signaling metabolites they generate. This project aims to identify the role of LOX5 and LOX10 in the response of *Zea mays* to HIPVs. We performed experiments in which fall armyworm (FAW) are allowed to feed on wild-type maize seedlings resulting in the production of HIPVs. Such infested plants are termed “emitters”. The *lox* mutants exposed to emitter volatiles are termed “receivers”. We then collected leaf tissues from the receiver plants and quantified a large number of oxylipins and several major defense phytohormones via liquid chromatography tandem mass spectrometry (LC-MS/MS). This analysis revealed significant perturbations in metabolite accumulation in several HIPV-induced oxylipins in *lox5* mutants. *lox10* mutants were deficient in a multitude of 13-oxylipins, confirming that it plays a major role in the HIPV response. Collectively, these results have revealed that both of these LOX isoforms play a significant role in HIPV-mediated defense priming against FAW.

DEDICATION

To Dr. Kissane, Dr. Kolomiets, Dr. Gorman, & Mr. Bennett who encouraged me to pursue research and gave me the opportunities to grow. To my caring and patient boyfriend and family who supported me through the research process with love and food.

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NOMENCLATURE

JA	Jasmonic acid
GLV	Green leaf volatile
HIPV	Herbivore-induced plant volatile
LOX	Lipoxygenase
WT	Wild-type
FAW	Fall armyworm

INTRODUCTION

Fall armyworms (FAW), *Spodoptera frugiperda*, represent a major pest of maize and other important agricultural crops around the world (Sparks, 1979). Infestation with these insects result in significant annual yield and economic losses of maize worldwide, and represent a growing problem (Sparks, 1979; Day et al., 2017). FAW are a chewing insect that can devastate maize yields; with a density of .2 to .8 larvae per plant, yields can be reduced by 5-20% (Capinera, 1999). One of the indirect ways plants defend themselves from this herbivory is through the emission of herbivore-induced plant volatiles (HIPVs), which can attract predatory insects that kill the herbivore (Farag & Pare, 2002; Heil, 2008). These volatiles are essentially a language, through which plants communicate between systemic tissues of a single plant, as well as between different plants. Diverse types of HIPVs from various volatile organic compound (VOC) groups can be produced by plants at different points after herbivory (Erb et al., 2015). One of the earliest groups of HIPVs released after herbivory are green leaf volatiles (GLVs), which are known to induce the synthesis of jasmonic acid (JA), phytohormone that is required for defense against insects (Engelberth et al., 2004; Erb et al., 2015). JA is synthesized by LOXs and is the best characterized oxylipin, but there are hundreds of other oxylipins that remain uncharacterized. Oxylipins are synthesized in the LOX pathway which begins with the oxygenation of polyunsaturated fatty acids, primarily linoleic (C18:2) and linolenic (C18:3), by LOXs to generate lipid hydroperoxides. 9-LOX and 13-LOX pathways generate 9- and 13-hydopexides respectively, and these hydroperoxides then enter the allene oxide synthase (AOS), hydroperoxide lyase (HPL), peroxygenase (POX), reductase (RED), LOX, epoxy alcohol

synthase (EAS), or divinyl ether synthase pathways that collectively lead to the synthesis of a large number of structurally and functionally diverse oxylipins.

The AOS pathway is best known for its synthesis of JA, including its precursor, 12-OPDA, and its biologically active derivative, JA-Ile (Fonseca, et al., 2009). Only a small amount of 12-OPDA is converted to JA (Gorman et al., 2020), and it has its own signaling activity distinct from JA (Wang et al., 2020a; Dave & Graham, 2012). The AOS pathway also produces α- and γ-ketols, lesser-known metabolites that have gained recent traction as important long-distance systemic signals of induced systemic resistance (ISR) in maize (Wang et al., 2020a; Wang et al., 2020b). The HPL produces the GLVs as well as their 12-carbon counterparts, traumatins, which are known to induce widespread transcriptomic responses in plants (Bonaventure et al., 2011). The RED pathway produces lipid hydroxides that are involved in defense against pathogens and insects (Prost et al., 2005; Marcos et al., 2015; Vellosillo et al., 2013). The POX pathway also makes some of the same lipid hydroxides and also makes various lipid epoxides and dihydroxides. The LOX pathway is responsible for epoxy-keto-octadecanoates, as well as 5-carbon volatiles and their 13-carbon counterparts (Salch et al., 1995). The EAS pathway makes epoxides in trihydroxides but the role of these compound in plants are not well understood (Borrego & Kolomiets, 2016). The role of metabolites produced by the divinyl ether synthase pathway is also not well understood. Oxylipins as a whole are known to be involved in the regulation of many different aspects of plant physiology, including defense.

While the mechanisms behind plant perception of HIPVs are still poorly understood, previous work has shown that HIPVs induce potent responses in plants. Engelberth et al. (2004) showed that when maize seedlings perceive the presence of GLVs, they synthesize greater levels

of JA. In maize, GLVs are synthesized by a single LOX gene, LOX10, and it is critical in defense against FAW (Rojas et al., 2018; Christensen et al., 2013). Exposure of uninfested plants, receivers, to HIPVs from infested plants, emitters, also is known to trigger increased synthesis of other metabolites, including sesquiterpenes – a group of defense-related metabolites (War et al., 2011). Constantino (2017) showed that GLVs, which are solely derived from LOX10 substrate, are produced in response to pathogen infection of maize and that they induce JA accumulation in a LOX5-dependent manner. Given that LOX5 is a tonoplast-localized 9-LOX (Tolley et al., 2018), this study suggests that LOX5 makes an important 9-oxylipin signal that is capable of stimulating JA biosynthesis, and implicates LOX5 as an important gene for defense against insect herbivory. This also implicates LOX10 as an important gene in emitter plants, and an important contributor to HIPVs.

We utilize knockout mutant maize lines of both LOX5 and LOX10 to better elucidate the role of LOXs in HIPV-mediated priming. As LOX10 is the only LOX capable of GLV synthesis, *lox10* mutants are devoid of GLVs and we specifically use this mutant for studying the effects of GLVs on the receiver metabolic responses to overall HIPV blends. By comparing the metabolic response of *lox5* and *lox10* maize mutants to wild-type (WT) receivers, we determine the role of these genes in metabolic response to FAW-induced HIPVs. GLVs were found to be the specific aspect of the HIPV blend that induces the synthesis of JA. Furthermore, we found that while LOX10 is required for induction of various metabolites in the receiver plant, it is not required for the induction of JA in response to HIPVs. We also found that LOX5 is critical for induction of several defensive secondary metabolites in response to HIPVs, in particular JA-Ile. These results show that LOXs are involved in HIPV-mediated priming and may constitute critical regulators for preemptive defense against herbivory.

METHODS

2.1 Plant and Insect Material

PCR screening of the *Mutator*-transposon insertional genetics resource at DuPont-Pioneer, Inc. (http://www.pioneer.com) for insertions in ZmLOX5 and ZmLOX10 procured mutant alleles of these genes (Yan et al., 2012; Christensen et al., 2013). After confirmation that the *lox5-3* and *lox10-3* alleles were exon-insertional knockout mutants, they were backcrossed into the B73 inbred line to the backcross 7 stage (98.5.% genome identity to the recurrent inbred parent line). Plants were grown on light shelves (~300 µmol m^{-2} s^{-1}) under a 14 h (light):10 h (dark) light regime to the V3 developmental stage in TX-360 Metro Mix soil (Sun Gro Horticulture, Agawam, MA) 14 h of light (~300 µmol m^{-2} s^{-1}). FAW were hatched and reared on cornmeal agar diet for 14 days, under the same conditions as described above.

2.2 Volatile Analysis

WT plants in the B73 genetic inbred background were grown to the V4 stage in a growth chamber as described above before being placed into 6-L glass containers, 10 plants per jar/replicate. Approximately five 3rd instar FAW were placed on each plant to elicit HIPV emissions, whereas controls remained uninfested. Volatiles were collected onto HaySepQ filter traps containing 80-100 mesh (Supelco, Bellefonte, PA) via dynamic airflow (approximately 1 L/min) for 5 h, during which FAW were allowed to freely roam and feed. Volatiles were eluted off the HaySepQ filter traps with 250 µL of dichloromethane containing 100 µM of the internal standard, (4*Z*)-hexenol (Sigma-Aldrich, St. Louis, MO).

An Agilent 7890B gas chromatograph connected to an Agilent 5977B quadrupole mass spectrometer (Agilent, Santa Clara, CA) was utilized to quantify volatiles in these samples. Two

µL of liquid sample was injected splitless into a HP-5ms Ultra Inert column (Agilent, Santa Clara, CA). The inlet temperature was set to 240 °C for the duration of the run. The oven temperature was as follows: 40 °C hold – 2 min, 3 °C/min ramp to 160 °C, 15 °C/min ramp to 280 °C, 280 °C/min hold – 2 min. The solvent delay was 2.5 min. Analytes were fragmented by positive EI (230 °C – source, 150 °C – quadrupole, ionization energy – 70eV, scan range – 25-500 amu). Most compounds were identified and quantified based off of retention times and spectra of pure external standards purchased from Sigma-Aldrich (St. Louis, MO). 4-oxo-(2E)-hexenal was identified based off matching of mass spectra and retention index (RI), calculated according to Van Der Dool and Kratz (1963). All volatiles were quantified based on utilizing internal and external standards.

2.3 HIPV Exposure

B73 inbred wild-type line (WT) and *lox* mutants in the B73 background were exposed to HIPVs produced from B73 and *lox10* mutants infested with FAW or volatiles emitted by uninfested B73 for 5 hours. The plants that produced either control volatiles or HIPVs are termed "emitters", and the plants exposed to the volatiles are referred to as the "receivers". Six emitters and 6 receivers per treatment were enclosed within separate glass jars (~6 L) connected through tubing that utilized unidirectional air flow towards the receivers. Unidirectional air flow was generated using vacuum lines to slightly pull air out of the jar of the receivers, which in turn will cause headspace air from the emitters' jar to be dynamically pulled into the receivers' chamber. Six emitter plants had FAW placed on them (infested), and six had no FAW placed on them (control). Infestation of plants occurred by placing 5 individual 14-day-old FAW on each plant, which were allowed to freely roam and feed for 5 h. After 5 h of exposure, the 2nd and 3rd leaves

of the receivers were harvested and immediately frozen in liquid N_2. Samples were then stored at -80 ^{o}C until further use.

2.4 Metabolite Analysis

Samples were ground using a mortar and pestle in liquid nitrogen and approximately 100 mg was then used for extraction of metabolites. Hormones were extracted from tissue and quantified by LC-MS/MS. One hundred mg of ground tissue was mixed with 10 µL of 5 µM internal standards of d-JA (2,4,4-d_3; acetyl-2,2-d_2 JA (CDN Isotopes, Pointe-Claire, Quebec, Canada), d_6-SA (Sigma-Aldrich, St. Louis, MO), and 500 µL phytohormone extraction buffer (1-propanol/water/HCl [2:1:0.002 v/v/v]). The samples were agitated for 30 min at 4 °C in darkness and then 500 µL dichloromethane was added. The samples were then agitated for an additional 30 min at 4 °C in darkness and centrifuged at 17,000 x g for 5 min. The lower organic layer of each sample was transferred to a glass vial for evaporation under nitrogen gas. Samples were resuspended in 150 µL methanol, transferred to a 1.5 mL microcentrifuge tube and stored at -20 °C for 2 days. Samples were then centrifuged at 17,000 x g for 2 min to pellet debris. Ninety µL of supernatant was transferred into autosampler vials for LC-MS/MS analysis. The simultaneous detection of phytohormones and oxylipins utilized methods of Muller et al. (2011) with modifications. An Ascentis Express C-18 Column (3 cm × 2.1 mm, 2.7 µm) (Sigma-Aldrich) connected to an API 3200 LC-MS/MS (Sciex, Framingham, MA) using electrospray ionization with multiple reaction monitoring. The injection volume was 10 µL and had a 450 µL min-1 mobile phase consisting of Solution A (0.2% acetic acid in water) and Solution B (0.2% acetic acid in acetonitrile) with a gradient consisting of (time – %B): 0.5 – 10%, 1.0 – 20%, 21.0 – 70%, 24.6 – 100%, 24.8 – 10%, 29 – stop. All hormones were quantified by comparing against isotopically-labeled internal standards from Sigma-Aldrich and Cayman Chemical (Ann Arbor,

MI). Full chemical names and abbreviations of compounds measured in this analysis can be found in Table 2.1.

Table 2.1: List of metabolites measured in LC-MS/MS analysis. Table shows the type of lipid substrate, class of LOX, and pathway for each metabolite. Shows full chemical name as well as names used in figures.

Class	Pathway	Compound	Lipid	Chemical/Common Name
9-LOX	RED/POX	9-HOD	18:2	9(*S*)-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid
		9-HOT	18:3	9(*S*)-hydroxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid
	POX	9,10-EpOM	18:2	cis-9,10-expoxy-12(*Z*)-octadecenoic acid ***/*** coronaric acid
		9,10-EpOD	18:3	cis-9,10-expoxy-12(*Z*),15(Z)-octadecenoic acid
		9,10-diHOM	18:2	threo-9,10-dihydroxy-12(*Z*)-octadecenoic acid
	LOX	9-KOD	18:2	9(*S*)-oxo-10(*E*),12(*Z*)-octadecatrienoic acid
		9-KOT	18:3	9(*S*)-oxo-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid
	AOS	9,10-KOMA	18:2	9-hydroxy-10-oxo-12(*Z*)-octadecadienoic acid
		9,10-KODA	18:3	9-hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic acid
		13,10-KOMA	18:2	13-hydroxy-10-oxo-11(*E*)-octadecadienoic acid
		13,10-KODA	18:3	13-hydroxy-10-oxo-11(*E*),15(*Z*)-octadecadienoic acid
		10-OPEA	18:2	10-oxo-11(*Z*)-phytodienoic acid
		10-OPDA	18:3	10-oxo-11(*Z*),15(*Z*)-phytodienoic acid
	EAS	9,12,13-THOM	18:2	9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoic acid
		9,12,13-THOD	18:3	9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*),15(*Z*)-octadecadienoic acid
		9,10,11-THOM	18:2	9(*S*),10(*S*),11(R)-trihydroxy-12(*Z*)-octadecenoic acid
		9,10,11-THOD	18:3	9(*S*),10(*S*),11(R)-trihydroxy-12(*Z*),15(*Z*)-octadecadienoic acid
	HPL	AZA	18:2/3	nonanedicarboxylic acid ***/*** azelaic acid
13-LOX	RED/POX	13-HOD	18:2	13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecatrienoic acid
		13-HOT	18:3	13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid
		11-HHT	16:3	11(*S*)-7(Z),9(*E*),13(*Z*)-hexadecetrienoic acid
	POX	12,13-EpOM	18:2	cis-12,13-expoxy-9(*Z*)-octadecenoic acid
		12,13-EpOD	18:3	cis-12,13-expoxy-9(*Z*),15(Z)-octadecenoic acid
		12,13-diHOME	18:2	threo-12,13-dihydroxy-9(*Z*)-octadecenoic acid
	LOX	13-KOD	18:2	13-oxo-9(*Z*),11(*E*)-octadecadienoic acid
		13-KOT	18:3	13-oxo-9(*Z*),11(*E*),15(Z)-octadecadienoic acid
		12,13-Ep-9KOM	18:2	12,13-epoxy-9-oxo-10(*E*)-octadecenoic acid
		OTD	18:3	13-oxo-9(*Z*),11(*E*)-tridecadienoic acid
	AOS	9,12-KOMA	18:2	9-hydroxy-12-oxo-10(*E*)-octadecenoic acid
		9,12-KODA	18:3	9-hydroxy-12-oxo-10(*E*),15(*Z*)-octadecadienoic acid
		13,12-KOMA	18:2	13-hydroxy-12-oxo-9(*Z*)-octadecenoic acid
		13,12-KODA	18:3	13-hydroxy-12-oxo-9(*Z*),15(*Z*)-octadecadienoic acid
		12-OPEA	18:2	12-oxo-10(*Z*)-phytodienoic acid
		12-OPDA	18:3	12-oxo-10(*Z*),15(*Z*)-phytodienoic acid
		dn12-OPDA	16:3	dinor-12-oxo-10(Z),15(Z)-phytodienoic acid
		OPC-8	18:3	8-[3-oxo-2-cis-[(Z)-2-pentenylcyclopentyl]octanoic acid
		OPC-4	18:3	8-[3-oxo-2-cis-[(Z)-2-pentenylcyclopentyl]butanoic acid
		JA	18:3	(+)-7-iso-jasmonic acid
		JA-Ile	18:3	(+)-7-iso-jasmonic acid isoluecine
		12COOH-JA-Ile	18:3	12-carboxy-jasmonic acid

Table 2.1: List of metabolites measured in LC-MS/MS analysis continued.

Class	Pathway	Compound	Lipid	Chemical/Common Name
13-LOX	AOS	12OH-JA	18:3	12-hydroxy-jasmonic acid
		12OH-JA-Ile	18:3	12-hydroxy-jasmonic acid isoluecine
		CCA	18:3	curcurbic Acid
		DH-JA	18:3	9,10-dihydro-jasmonic acid
	EAS	9,12,13-THOM	18:2	9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoic acid
		9,12,13-THOD	18:3	9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*),15(*Z*)-octadecadienoic acid
		9,10,13-THOM	18:2	9(*S*),10(*S*),13(*S*)-trihydroxy-11(*E*)-octadecenoic acid
		9,10,13-THOD	18:3	9(*S*),10(*S*),13(*S*)-trihydroxy-11(*E*),15(*Z*)-octadecadienoic acid
	HPL	9OH-TAN	18:3	9-hydroxy-12-oxo-10(*E*)-dodecenoic acid **/** 9-hydroxy-traumatin
		9Z-TAN	18:2/3	12-oxo-9(*Z*)-dodecenoic acid
		TA	18:2/3	2(*E*)-dodecenedioic acid **/** traumatic acid
		TAN	18:2/3	12-oxo-10(*E*)-dodecenoic acid **/** traumatin
Other	*a*-DOX	2OH-PA	16:3	2-hydroxy-hexadecanoic acid **/** 2-hydroxy-palmitic acid
		10-HOD	18:2	10-hydroxy-8(*E*),12(*Z*)-octadecadienoic acid
		2-HOD	18:2	2-hydroxy-9(*Z*),15(*Z*)-octadecadienoic acid
	PAL	CA	NA	cinnamic acid
		COUMA	NA	coumaric acid
		BA	NA	benzoic acid
	PAL/ICS	SA	NA	salicylic acid
	MEP	ABA	NA	abscisic acid

RESULTS

3.1 GLVs are the Major Class of Volatile Comprising FAW-induced Maize HIPVs

We first wanted to better determine which HIPVs are emitted in B73 WT in response to FAW herbivory, so we placed plants in large chambers and either placed FAW on them or not (control). We allowed FAWs to feed for 5 h, during which volatiles were continuously collected onto a volatile trap. After analyzing the collected volatiles, we found that uninfested maize largely emitted terpenes, with smaller amounts of GLVs, pentyl leaf volatiles (PLVs), and other miscellaneous volatiles present (Figure 3.1a). However, GLV emissions in B73 infested with FAW were much higher, and constituted the majority of volatiles emitted in response to herbivory (Figure 3.1b,c). Interestingly, (2*E*)-hexenal was emitted in high amounts after herbivory and was equal to the amount of (3*Z*)-hexenal (Figure 3.1c). Previous analysis of maize volatiles has shown that (3*Z*)-hexenal is emitted in high amounts compared to (2*E*)-hexenal, which is typically lowly emitted in maize (He et al., 2020; Gorman et al., 2020). Previous work with lepidopterans revealed that some species possess an (3*Z*:2*E*)-hexenal isomerase that facilitates synthesis of (2*E*)-hexenal (Allman et al., 2010), and it seems that FAW also possess a similar isomerase. Several PLVs were also increased in response to FAW herbivory (Figure 3.1e). Terpenes and other volatiles were major components of uninfested plant volatile blends, but were more lowly emitted by infested plants and made up a much smaller proportion of the volatile blend (Figure 3.1a,b,f). Volatiles belonging to other miscellaneous groups were largely not impacted, with only levels of triacetin being lower in infested plants (Figure 3.1d). Overall, this experiment shows that GLVs are the major inducible group of FAW-induced HIPV emissions in maize, and suggest their importance in mediating priming against FAW herbivory.

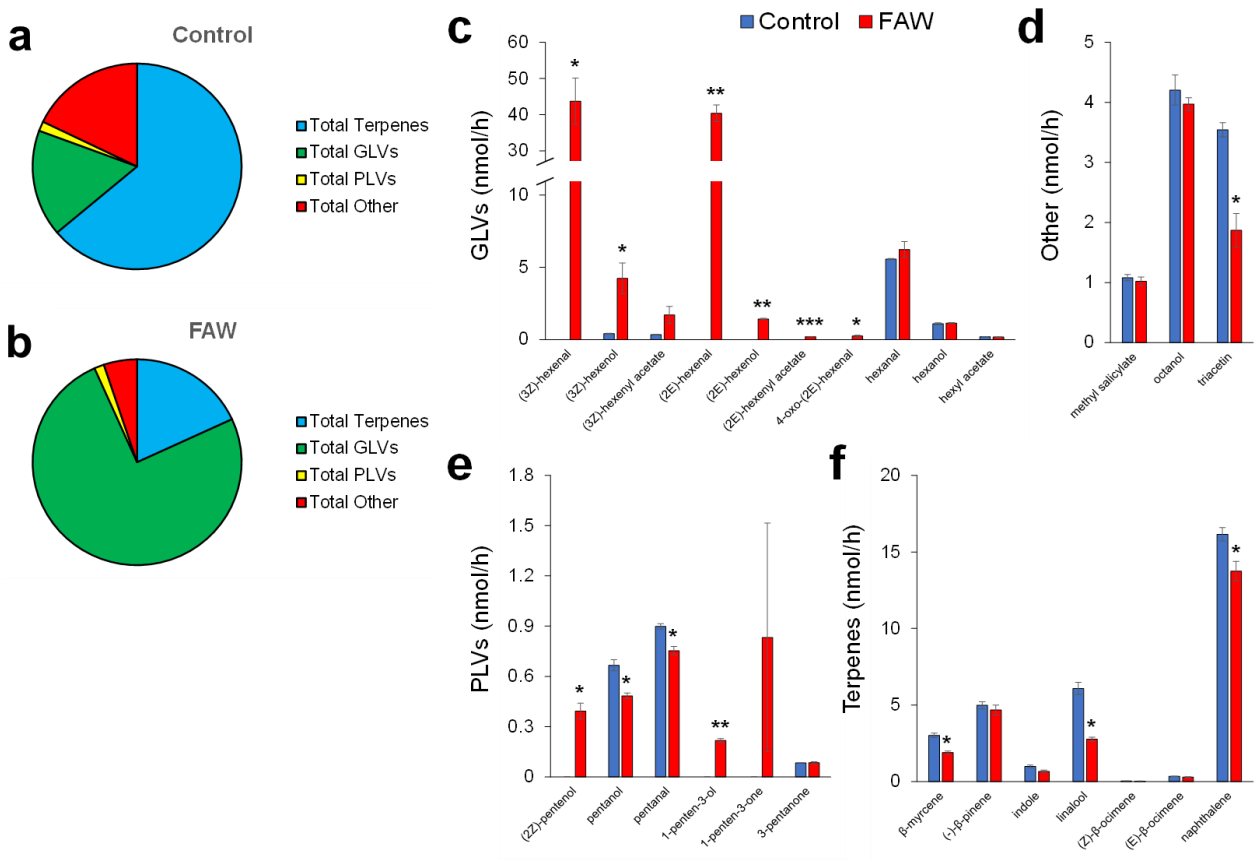

Figure 3.1: Volatile emissions of WT B73 maize in response to herbivory by FAW. (a-b) show proportion of major volatile classes in volatile blends of uninfested (a) and (b) FAW infested plants. (c-f) show the amount of individual volatiles emitted (in nmol/h) by uninfested (blue bars) and FAW infested plants (red bars), with GLVs shown in (c), miscellaneous volatiles in (d), pentyl leaf volatiles (PLVs) in (e), and terpenes in (f).

3.2 GLVs are the Specific HIPVs Responsible for JA Induction, LOX10 is Required for Induction and Suppression of Many HIPV-modulated Oxylipins

In order to ascertain the role of GLVs in the overall HIPV blend, we exposed WT to volatiles from WT and GLV-deficient *lox10* mutants that were either infested or uninfested with FAW. We also sought to elucidate the role of LOX10 in receiver plants, therefore, we also exposed *lox10* mutants to volatiles from WT infested or uninfested with FAW. We profiled a diverse array of metabolites using LC-MS/MS and constructed a heatmap that highlights the

relative quantities of these metabolites across every treatment/genotype. We found several different metabolites that accumulated differentially depending on the genotype of the emitter/receiver as well as the treatment of the emitter. This heatmap displayed that LOX10 was a major LOX isoform involved in the synthesis of a diverse array of oxylipins and other metabolites in receiver plants (Figure 3.2). This also revealed that many metabolites are specifically induced by HIPVs from FAW infested plants. By evaluating the impact of *lox10* mutant-HIPVs relative to the impact of WT-HIPVs on WT receivers, this analysis also revealed specific metabolites that are explicitly induced by GLVs in the HIPV blend.

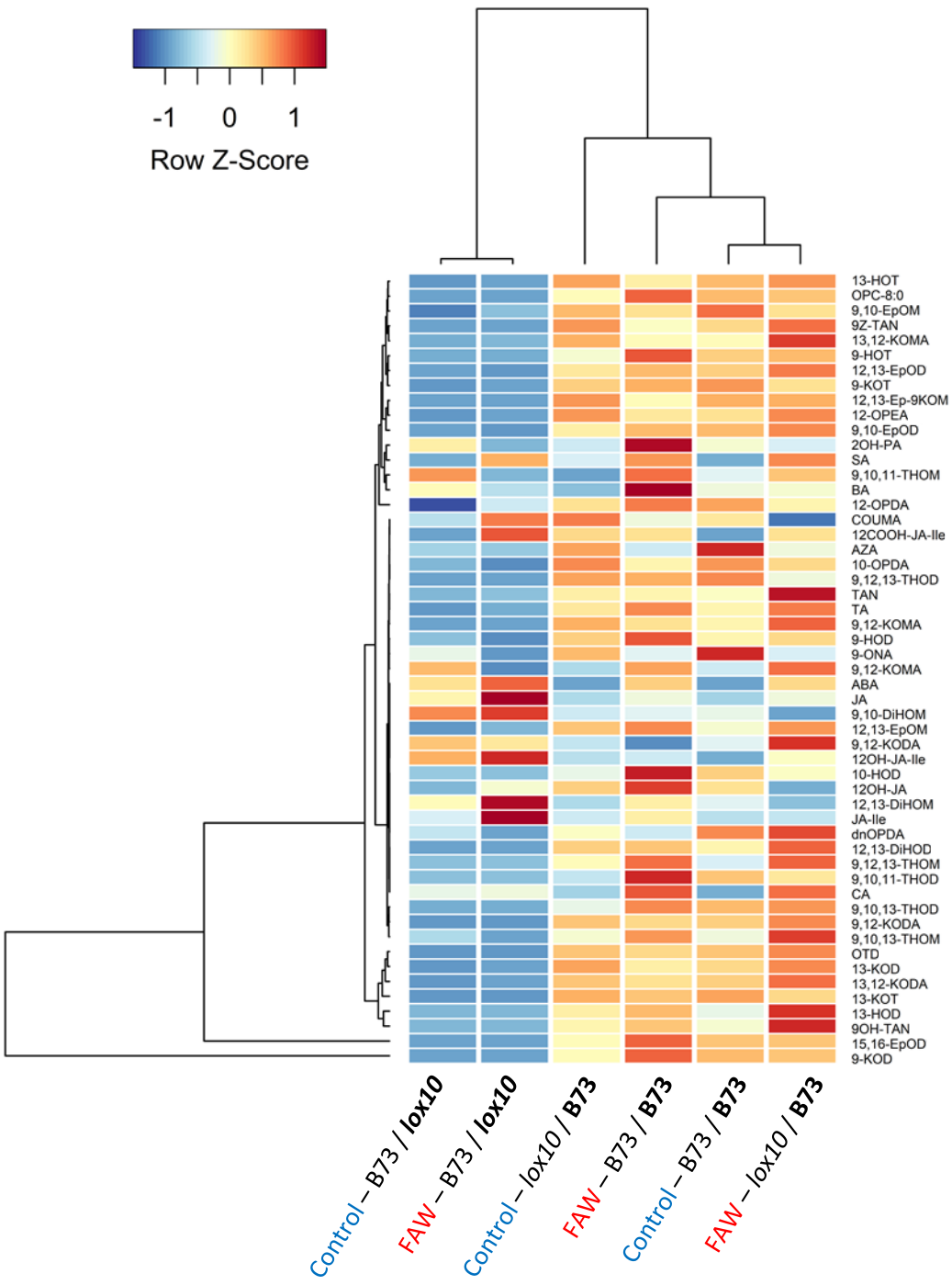

Figure 3.2: Heatmap of the HIPV-mediated changes in the accumulation of metabolites in the receiver plants). Both metabolites and genotype/treatment combiniations are grouped in terms of similarity. Relative quantites of metabolites are shown, with red indicating higher amounts and blue indicating lower quantites.

We compared the relative responses of the WT receivers exposed to *lox10* mutant volatiles (*lox10*-WT), both control and FAW-infested, and WT exposed to WT volatiles (WT-WT), both control and FAW-infested. This allowed us to determine which metabolite responses were specifically related to GLV-mediated signaling. The jasmonates, JA and JA-Ile, as well as 2OH-palmitic acid (2OH-PA) were identified as metabolites specifically induced by GLVs (Figure 3.3). These metabolites were induced in WT by HIPVs from WT that were infested by FAW, but not in WT exposed to control WT volatiles, nor WT exposed to *lox10* mutant volatiles. This was true of WT exposed to both control and FAW-induced HIPVs from lox10 mutants, confirming that other HIPVs are not involved in jasmonate response.

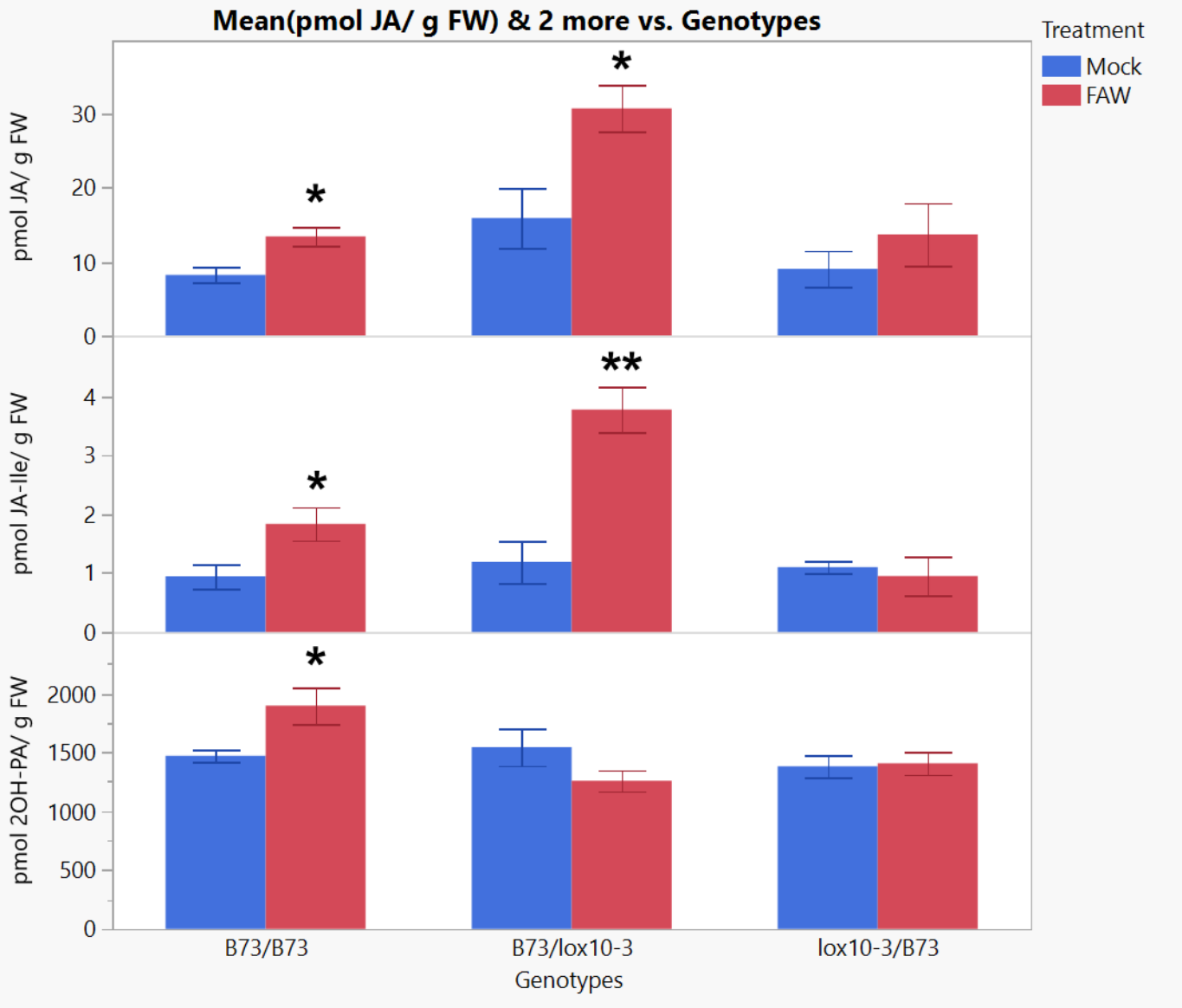

Figure 3.3: Metabolies regulated specifically by GLVs. The blue bar represents the mock treatment while the red bar represents the FAW treatment. The genotype of the emitter is on the left of the slash and the reciever on the right. Asteriks indicate level of signifigance[p<.05=(*), p<.005=(**)]. Error bars shown.

To evaluate the role of LOX10 in metabolite induction in response to HIPVs, *lox10* mutants were also used as receivers. Some metabolites that were HIPV-inducible in WT were not induced in *lox10* mutants. These metabolites are derived from diverse metabolic pathways.

These metabolites include the salicylic acid (SA) precursors, cinnamic acid (CA) and benzoic acid (BA), which are produced in the phenylpropanoid pathway (Figure 3.4). From the EAS pathway, the trihydroxides, 9,12,13-THOM and 9,10,13-THOM, followed a similar pattern of accumulation. Two additional metabolites, 12,13-EpOM, from the POX pathway, and 13-HOD, from both the POX and RED pathways mirrored this pattern. The two 9-oxylipins, 9-HOD and 9-HOT, also followed suit but did not reach statistical significance.

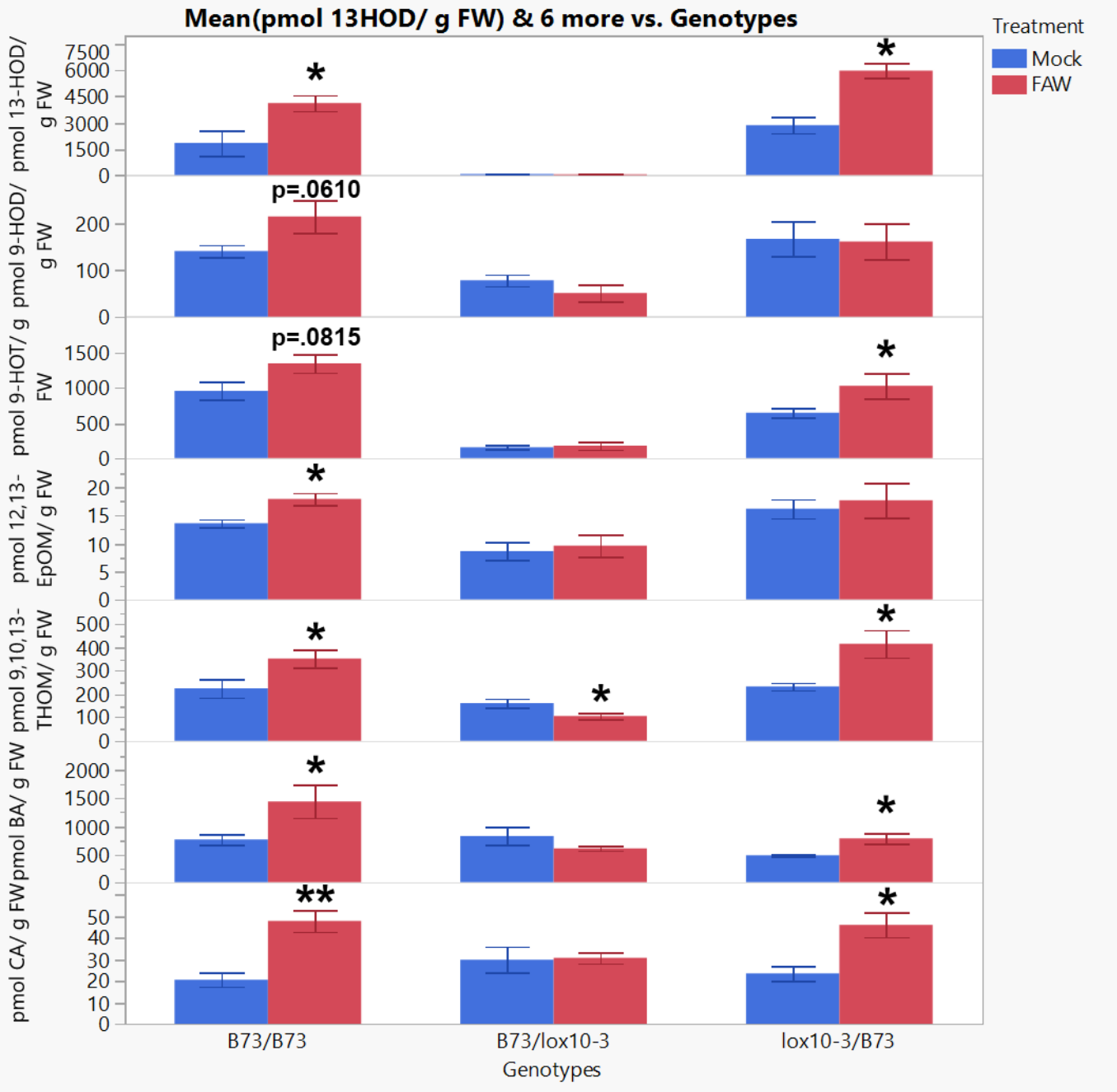

Figure 3.4: Metabolites induced in response to HIPVs in WT, but not in lox10. The blue bar represents the mock treatment while the red bar represents the FAW treatment. The genotype of the emitter is on the left of the slash and the reciever on the right. Asteriks indicate level of signifigance[p<.05=(*), p<.005=(**)]. Error bars shown.

Oppositely, some metabolites accumulated in *lox10* mutant receivers of HIPVs relative to control volatiles despite there being no induction from HIPVs in WT receivers. In particular, mostly oxylipins produced in the 13-AOS pathway were increased, including the jasmonates, 12-OPDA and 12OH-JA, an inactive form of JA (Figure 3.5). Other AOS products, collectively referred to as ketols, were also increased in *lox10* mutants exposed to HIPVs. This included, 9,12-KODA, 13,12-KODA, and 13,12-KOMA. Some of these metabolites, were not statistically significant, but were close ($p<.09$). Coumaric acid (COUMA), a metabolite produced in the phenylpropanoid pathway and an SA precursor, was also induced in the *lox10* mutant receivers of HIPVs. These results suggest that LOX10 not only plays an important role in the induction of metabolites in response to HIPVs, but also plays an active role in the suppression of certain metabolites as well. These results shed new light into the role of the major maize 13-LOX isoform, LOX10, in HIPV-mediated priming. To expand upon this, we also wanted to investigate the role of LOX5, a 9-LOX isoform known to be involved in the regulation of JA biosynthesis and FAW defense.

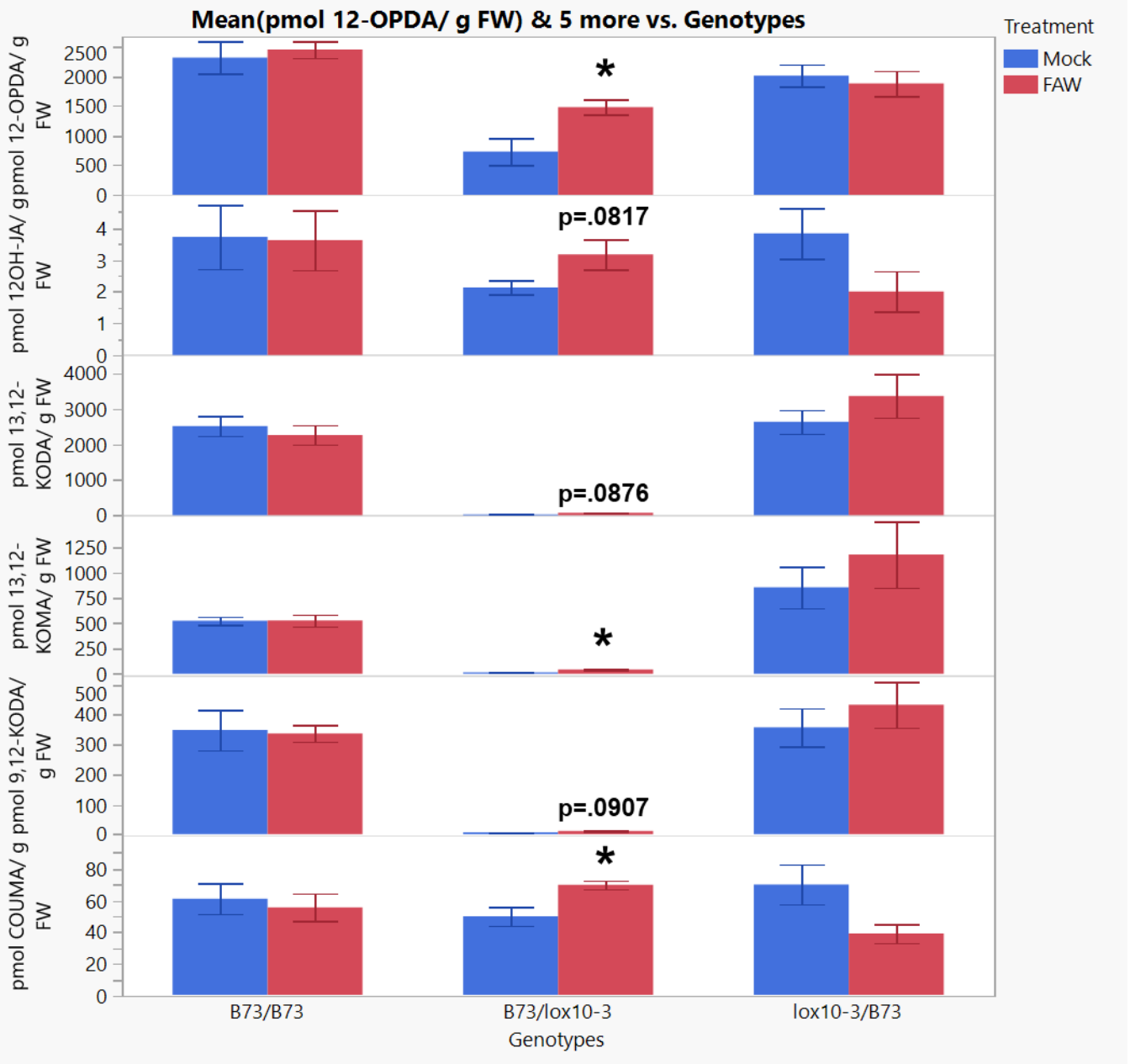

Figure 3.5: Metabolites induced to higher levels in lox10 receiver exposed to HIPVs compared to uninfested VOCs. The blue bar represents the mock treatment while the red bar represents the FAW treatment. The genotype of the emitter is on the left of the slash and the reciever on the right Asteriks indicate level of signifigance[p<.05=(*), p<.005=(**)]. Error bars shown.

3.3 LOX5 is Required for the Induction of Several Metabolites, Including JA-Ile

In order to investigate the role of LOX5 in response to HIPVs, we exposed both WT and *lox5* mutant receivers to volatiles from WT either infested with FAW or not. By comparing the responses to FAW and mock treatment in each of the receivers, we were able to elucidate several metabolites that functional LOX5 either induces or suppresses in response to HIPV perception. As we did previously, we constructed heatmaps to observe the overall impact of LOX5 on HIPV-mediated metabolite responses (Figure 3.6).

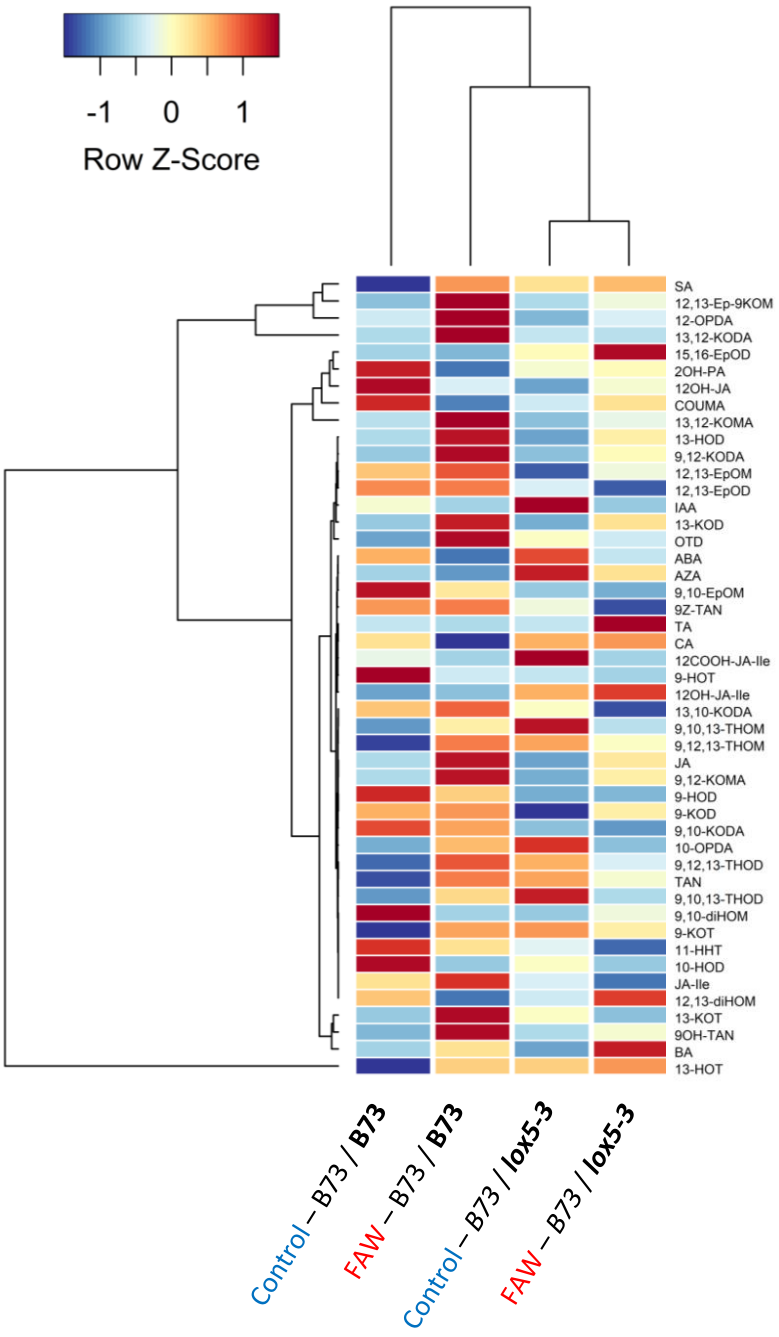

Figure 3.6: Heatmap of the LOX5-dependent changes in the accumulation of metabolites in the receiver plants. Grouped according to clustering. As the legend key of the heatmap indicates, the darker shade of red equates to a higher induction of the metabolite wheras the darker shade of blue indicates less induction.

The most notable observation in this experiment involved JA-Ile, the most biologically active form of JA. We found that while JA-Ile was induced in WT in response to HIPVs, it became undetectable in *lox5* mutant receivers after exposure to HIPVs (Figure 3.6, Figure 3.7). Furthermore, the 13-oxylipins, 9,12,13-THOM, an EAS product, and TAN, a HPL product, both showed a significant lack of accumulation in *lox5* mutants exposed to HIPVs but not in WT. 12-OPDA and the EAS products, 9,10,13-THOD and 9,12,13-THOD, were other oxylipins that showed similar trends (Figure 3.7). Lastly, SA also was increased in WT without a matching response in *lox5* mutant receivers (Figure 3.7). This experiment provides evidence that LOX5 contributes to not only the induction of several oxylipins, but also regulated the synthesis of the 13-LOX product, JA-Ile, in response to HIPVs. These experiments demonstrate the importance of not only 13-LOXs in HIPV-mediated metabolite responses, but also the relevance of 9-LOXs in this process.

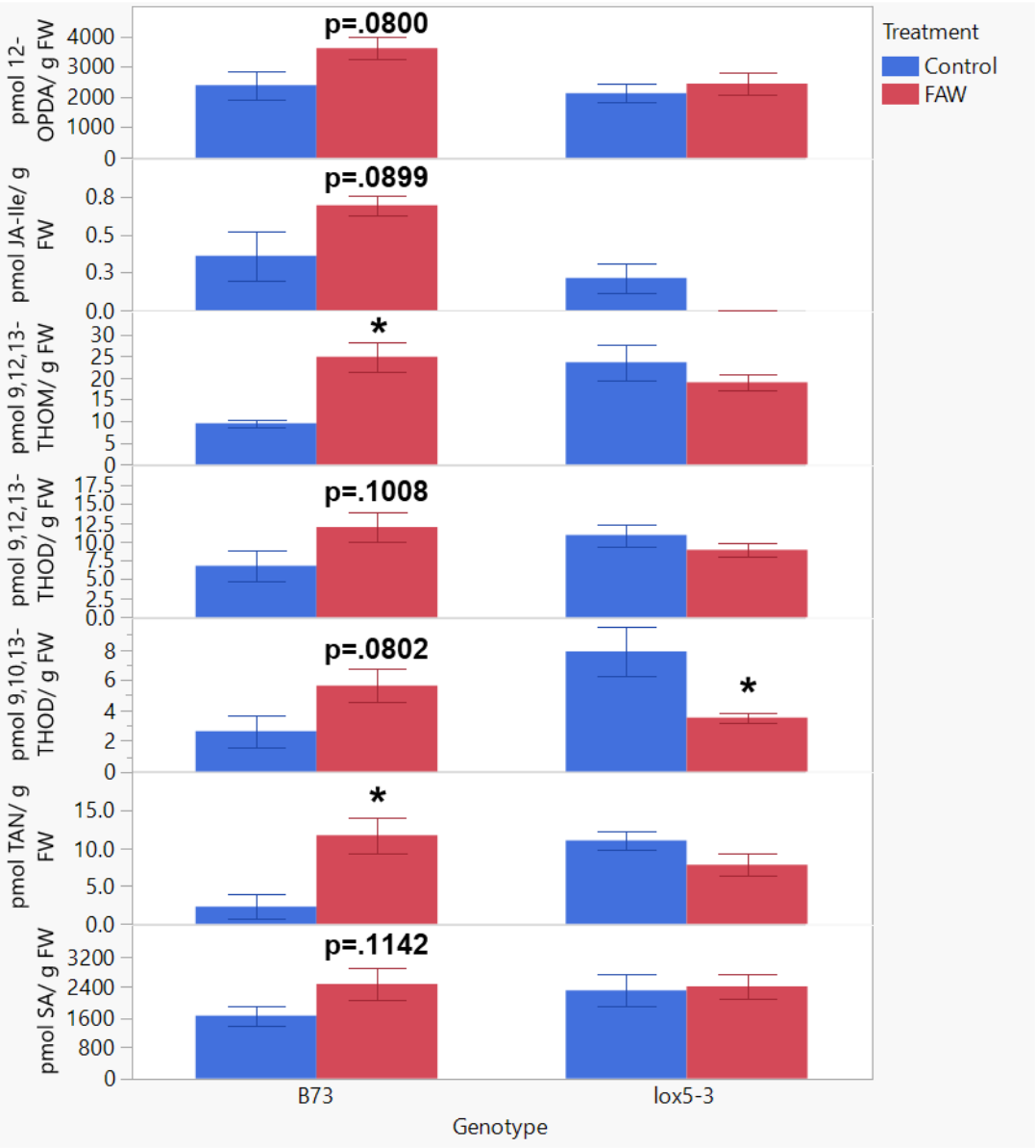

Figure 3.7: Metabolites not induced by HIPVs in the lox5 recievers. The blue bar represents the mock treatment while the red bar represents the FAW treatment. Asteriks indicate level of signifigance[p<.05=(*), p<.005=(**)]. Error bars shown.

DISCUSSION AND CONCLUSION

To elucidate the roles of LOX5 and LOX10 in HIPV-mediated priming, knockout mutants of both genes were used as receivers in these experiments. The metabolic responses of the receiver plants after exposure to HIPVs or control volatiles were compared to gain an understanding of these LOXs' role in metabolite response. Furthermore, *lox10* mutants were also used as emitters in order to understand the specific role of GLVs within the overall HIPV blend, with regard to metabolite response in receivers. LOX10 was chosen for this study because it is the sole LOX isoform responsible for GLV synthesis and is the most abundant 13-LOX in leaves (Christensen et al., 2013). LOX5 was selected for its known induction of JA in response to pathogen induced plant volatiles (Constantino, 2017). Furthermore, LOX10 is known to be an important regulator of defense against FAW in maize (Rojas et al., 2018). Though GLVs are a known critical component of plant indirect defense against insect herbivory, their specific role in HIPV blends to induce defenses in neighboring receiver plants has yet to be fully elucidated. Furthermore, there was no information on the relative contribution of 9- or 13-LOXs to HIPV-mediated metabolite response.

Unsurprisingly, *lox10* mutant receivers showed much lower overall amounts of metabolites, even in response to control volatiles (Figure 3.2). This is in alignment with observations of He et al. (2020), which showed significantly impaired wounding responses of oxylipins, hormones and other metabolites in response to wounding. Also unsurprisingly, we found that GLVs in emitter plants were required for the induction of jasmonates, JA and JA-Ile in receiver plants (Figure 3.3). As these metabolites accumulated in WT in response to HIPVs, but not in response to WT control volatiles or *lox10* mutant volatiles. These results suggest that

GLVs are the most important class of HIPV emitted by FAW-infested maize that induce JA accumulation.

JA is produced by the AOS pathway, and importantly, is derived exclusively from 13-hydroperoxides produced by 13-LOXs. JA itself does not possess any biological signaling activity (Fonseca et al., 2009). Instead, JA may be conjugated to a variety of amino acids or other functional groups to gain signaling activity (Borrego & Kolomiets, 2016). JA-Ile is the most biologically active form of JA and it is involved in insect defense signaling, Interestingly, the 9-LOX, LOX5, also seems to have some form of regulation of JA-Ile as *lox5* mutant receivers have decreased induction of JA-Ile in response to HIPV treatment compared to *lox5* mutants exposed to control volatiles (Figure 3.5). Given the relevance of JA-Ile to insect defense, this likely makes LOX5 and the oxylipin signals it produces highly relevant to HIPV-mediated defense priming. This also links GLVs produced by LOX10 to JA-Ile synthesis, mediated by LOX5, in neighboring plants. Like JA and JA-Ile, 2OH-PA also lacked a significant increase WT exposed to control volatiles or HIPVs from *lox10* emitters (Figure 3.3). 2OH-PA is a derivative of palmitic acid (16:0), synthesized by fatty acid hydrolases (Nagano et al., 2012). Currently, there is no evidence as to the role of 2OH-PA in insect defense, however these results indicate that it could be of potential relevance.

Several oxylipins induced in WT in response to HIPVs lacked corresponding response in *lox10* mutant receivers. Many of these oxylipins were derived from the 13-LOX pathway, such as 12,13-EpOM, 9,10,13-THOM, and 13-HOD. Unfortunately, little is known about the biological roles of these metabolites, but it is likely they are involved in defense signaling as they are typically induced in response to stress (He et al., 2020). Interestingly, some 9-oxylipins responses were also deficient in *lox10* mutants exposed to HIPVs, including, 9-HOD and 9-HOT,

which are known to aid in defense against plant pathogens (Prost et al., 2005). Furthermore, 9-HOT also aids in the defense response to insects through cell wall modifications (Marcos et al., 2015; Vellosillo et al., 2013). Since LOX10 cannot directly synthesize these oxylipins, it is likely that LOX10 induces other 9-LOX for their synthesis, perhaps LOX5. The SA-precursors, CA and BA, also followed this pattern. SA has been suggested to aid insect defense in monocots (Qi et al., 2019), though the effects of its precursors on insect defense remains unknown.

Other metabolites accumulated in *lox10* mutant receivers of HIPVs relative to control volatiles despite there being no induction from HIPVs in WT receivers. The AOS produced jasmonates, 12-OPDA and 12OH-JA, were induced in *lox10* mutant receivers after exposure to HIPVs (Figure 3.4). In addition to being a precursor for JA synthesis, 12-OPDA is an important signaling molecule in its own right and is involved in induced systemic resistance in maize (Wang et al., 2020a), and resistance to aphids (Varsani et al., 2019). 12OH-JA, the inactive derivative of JA (Caarls et al., 2017), was also increased. This is likely a result of the plants trying to attenuate JA signaling in these plants, due to the high corresponding levels of JA in these plants. The AOS-derived ketols, 9,12-KODA, 13,12-KODA, and 13,12-KOMA, were also increased in *lox10* mutants exposed to HIPVs, though their overall amount was still far less than that of WT, due to *lox10* mutants possessing low basal levels of these metabolites (Het et al., 2020). Unfortunately, little is known about the 13-oxylipins, 13,12-KODA and 13,12-KOMA. The ketol, 9,12-KODA, acts as a priming agent for induction of ISR (Wang et al., 2020b). The phenylpropanoid product, COUMA, which is an SA precursor and aids in plant defense via cell wall biosynthesis (Tzin et al., 2017), was also elevated in *lox10* mutants in response to HIPVs.

While JA-Ile induction in response HIPVs was lacking in *lox5* mutants, it was not the only LOX5-dependant metabolite (Figure 3.7). The 13-oxylipins, 9,12,13-THOM and TAN, also both followed this trend. Alongside the lack of induction of 9,10,13-THOM by *lox10* mutants,

the lack of 9,12,13-THOM in *lox5* mutants suggests that the EAS pathway is important in the HIPV-mediated response. The HPL product, TAN, is a precursor for many derivatives that are involved in transcriptional regulation of many genes, and is thought to be involved in wounding responses (English et al., 1937; Bonaventure et al., 2011). Since TAN is the 12-carbon counterpart of GLVs, this suggests that HIPVs can correspondingly induce the synthesis and emission of GLVs in a LOX5-dependent manner. Ultimately, this suggests that GLVs, synthesized from LOX10, induce LOX5, which induces LOX10 in return. The EAS pathway again seemed to display its relevance to HIPV-mediated priming, with 9,12,13-THOD and 9,10,13-THOD responses appearing deficient in *lox5* mutants. Unfortunately, we were unable to evaluate the role of GLVs specifically on LOX5-medaited induction of metabolites, as we did not have enough plants to use *lox10* mutants as emitters with *lox5* mutant receivers. Future experiments should address this question. Other maize LOX isoforms, including the LOX2 (Constantino, 2017), which is induced by volatiles, and LOX8, which is involved in the bulk of wound-induced JA biosynthesis (Christensen et al., 2013). LOX4 should also be studied, due to its high homology with LOX5, 94% amino acid sequence identity (Park et al., 2010).

Collectively, these data have provided insight into the role of LOXs in HIPV mediated signaling, and shown that LOX5, a 9-LOX, can regulate JA-Ile synthesis in response to HIPVs. To the best of our knowledge, this is the first instance that a 9-LOX in any plant species has been shown to regulate JA synthesis involved in HIPV-mediated priming. Furthermore, this study has highlighted several interesting target defense metabolites, produced by both LOX5 and LOX10, for further study. To build upon this study, experiments are warranted to look into the role that these LOXs and identified metabolites play in insect defense after HIPV-mediated priming. The

findings of this study and future work are relevant to industrial agriculture as LOX-mediated insect priming is a promising sustainable alternative to pesticides (Pérez-Hedo, 2021).

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